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Insect cellular reactions to the lipopolysaccharide component of the bacterium *Serratia marcescens* are mediated by eicosanoids

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Abstract

Nodulation, which begins with the formation of cellular microaggregates, is the predominant cellular defense reaction to bacterial infections in insects. We suggested that these reactions to bacterial infections are mediated by eicosanoids. The lipopolysaccharide (LPS) component of some bacterial cells stimulates defense reactions in mammals and insects. Here, we report on experiments designed to test the hypothesis that eicosanoids mediate microaggregation reactions to LPS. Injections of LPS (purified from the bacterium, *Serratia marcescens*) into larvae of the tenebrionid beetle, *Zophobas atratus*, stimulated microaggregation reactions in a dose-dependent manner. Treatments with eicosanoid-biosynthesis inhibitors immediately prior to LPS challenge sharply reduced the microaggregation responses. Separate treatments with specific inhibitors of phospholipase A_2, cyclooxygenase and lipoxygenase reduced microaggregation, supporting our view that microaggregate formation involves lipoxygenase and cyclooxygenase products. The inhibitory influence of dexamethasone was apparent within 30 min after injection, and microaggregation was significantly reduced, relative to control insects, over the following 90 min. The dexamethasone effects were reversed by treating LPS-injected insects with the eicosanoid precursor, arachidonic acid. These findings indicate that cellular defense reactions to a specific component of bacterial cells are mediated by eicosanoids, and open up new possibilities for dissecting detailed hemocytic actions in insect immune reactions to bacterial infections.

Keywords: Insect immunity; Eicosanoids; Lipopolysaccharide; *Zophobas atratus*

1. Introduction

Eicosanoid is a general term for all biologically active, oxygenated metabolites of three C20 polyunsaturated fatty acids, namely 20:3n-6, 20:4n-6 and 20:5n-3. Three main groups of eicosanoids are recognized (Stanley, 2000): (1) the prostaglandins and thromboxanes are products of the cyclooxygenase pathways; (2) epoxyeicosatrienoic acids are products of the so-called epoxygenase pathways; and (3) the lipoxygenase pathways are responsible for a large assemblage of products, including hydroxyeicosatetraenoic acids. These molecules serve as lipid mediators, which exert profound influences on cellular events in mammals. Indeed, eicosanoids are appreciated best in the contexts of their clinical importance in human and animal medicine.

In addition to their medical importance, recognition of the biological significance of eicosanoids in invertebrates is rapidly growing (Stanley and Howard, 1998; Stanley, 2000). Some eicosanoid actions in invertebrates seem to be limited to a relatively small number of species. For example, prostaglandins release egg-laying behavior in only a few species of insects (Stanley-Samuelson and Loher, 1986). Alternatively, it appears that other eicosanoid actions are quite basic to animal physiology, and these actions occur in most, if not all animals. This may be so for certain events in reproduction, in modulation of ion-transport phenomena and in cell-mediated host defense reactions (Stanley, 2000).

We have been considering the idea that eicosanoids mediate cellular defense reactions to bacterial infections in insects, and perhaps all invertebrates, that undertake cellular host defense actions. We first found that tobacco

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hornworms, *Manduca sexta*, that had been treated with eicosanoid-biosynthesis inhibitors prior to experimental infections were unable to clear bacterial infections from hemolymph circulation (Stanley-Samuelson et al., 1991). Microaggregation and nodulation are early cellular actions responsible for clearing large numbers of bacterial cells from circulation (Horohov and Dunn, 1983). In our second line of work, we found that these two cellular actions were sharply impaired in tobacco hornworms that had been treated with eicosanoid-biosynthesis inhibitors prior to experimental infections (Miller et al., 1994). Subsequent studies revealed that eicosanoids mediate microaggregation and nodulation reactions to bacterial infections in other insect species, including the larvae of the beetle *Zophobas atratus* (Miller et al., 1996), true army worms, *Pseudaletia unipuncta*, black cutworms, *Agrotis ipsilon* (Jurenka et al., 1997), and silk moths, *Bombyx mori* (Stanley-Samuelson et al., 1997), all holometabolous insects. We also suggested that eicosanoids mediate cellular defense reactions to bacterial infections in adults of three hemimetabolous species, the cricket *Gryllus assimilis* (Miller et al., 1999) and the periodical cicadas, *Magicicada septendecim* and *Magicicada cassini* (Tunaz et al., 1999).

Eicosanoids mediate other aspects of insect immunity. Downer and colleagues suggested that eicosanoids mediate phagocytosis, cell spreading and the prophenyloxidase reactions to bacterial infections in larvae of the greater wax moth, *Galleria mellonella* (Mandato et al., 1997). Beyond these cellular reactions to infection, Morishima et al. (1997) found that eicosanoids mediate induction of two immune genes in the silkworm fat body, those for cecropin and lysozyme. Taken together, these results support our hypothesis that eicosanoids are important elements in the signal transduction processes that operate between a bacterial infection and the appearance of insect defense reactions.

We reported on the differential influence of four bacterial species on nodulation intensity for larvae of two insect species, *M. sexta* and *Z. atratus* (Howard et al., 1998). In both insects, we found that infections of two bacterial species, *Serratia marcescens* and *Escherichia coli*, resulted in high levels of nodulation, while identical infections with two other bacterial species, *Bacillus subtilis* and *Sarcina flava*, resulted in relatively low levels of nodulation. We inferred from these findings that insects are able to gage certain features of invading bacterial cells.

Outer features of bacterial cells, including peptidoglycan fragments and lipopolysaccharide, are involved in defense reactions to bacterial infections in vertebrates and invertebrates, and they are probably responsible for the differential nodulation reactions we observed. Peptidoglycan fragments induce synthesis of lysozyme in tobacco hornworms (Kanost et al., 1988). Similarly, LPS stimulates cellular aggregation and protein secretion (Charalambidis et al., 1995). On the basis of our earlier work, we hypothesized that eicosanoids mediate insect cellular reactions to LPS. In this paper we report the results of experiments designed to test this idea. Working with larvae of the tenebrionid beetle, *Z. atratus*, we found that injections of purified LPS resulted in the formation of microaggregations, a phase in nodulation. The microaggregation reactions were attenuated by pretreating the hornworms with eicosanoid-biosynthesis inhibitors.

2. Materials and methods

2.1. Insects

Late-stage larvae of the beetle *Z. atratus* were purchased from Nature's Way (Ross, OH) and maintained on a laboratory bench in terraria containing brewer's yeast, wheat flour and oatmeal, in a ratio of 1:16:128 (dry v/v). Water was provided by keeping damp towels on top of the mixture.

2.2. Preparing LPS from S. marcescens

There are several published protocols for preparing LPS, all of which yield LPS of differing purity. Here we describe an unpublished protocol used to yield pure LPS. *S. marcescens* (a wild-type strain provided by Dr Kenneth Nickerson, University of Nebraska, Lincoln) was grown to early stationary phase in Luria broth at the University of Nebraska fermentation facility. Cells were harvested by centrifugation and washed twice with deionized water and lyophilized. The dried cells constituted the starting material for the LPS extraction and purification.

Extraction and purification of LPS from *S. marcescens* involves four steps: delipidation, enzyme treatment, extraction of LPS and precipitation of LPS aggregates and, finally, ultracentrifugation.

2.2.1. Delipidation

Lyophilized cells were suspended at a ratio of 60 ml solvent per gram of lyophilized cells and extracted sequentially with continuous stirring with each of the following solvents: 95% ethanol (2 h); absolute acetone (2 h); and diethylether (1 h). Cells were centrifuged from each solvent prior to the addition of the next solvent in the series. Following extraction with diethylether the cells were allowed to dry in air overnight in a chemical hood.

2.2.2. Enzyme treatment

Dried, delipidated cells were suspended in deionized water at a ratio of 20 ml water per gram of initial lyophilized cells. DNAase and RNAase (Boehringer

Mannheim) were added to the solution at 1 mg each per 20 ml water, and then the solution was brought to 4 mM sodium azide. The mixture was incubated for 48 h at room temperature, after which 1 mg of protease K per 20 ml water was added and incubation continued at room temperature for 24 h. The entire suspension was added to a pre-wetted dialysis bag (Spectra/Por, MWCO:6-8000). The suspension was dialyzed at room temperature in 4 l of deionized water with stirring for 24 h. The water was changed five times in this period. Following dialysis the cell suspension was centrifuged for 1 h at 5000***g***. The resulting cell pellet/sediment was washed two times with absolute acetone and dried overnight.

2.2.3. LPS extraction and precipitation of LPS aggregates

The cells were suspended in phenol (10 ml of 90% phenol per gram of lyophilized cells measured at the stage of starting material). This mixture was stirred continuously at 50°C in an oil bath. After 24 h the mixture was cooled to room temperature, chloroform (10 ml per gram of lyophilized cells) and petroleum ether (16 ml per gram of lyophilized cells) were added to the phenol–cell suspension and stirring continued. After 30 min the mixture was centrifuged and the supernatant layer reserved. The sediment was re-extracted with 90% phenol/chloroform/petroleum ether with stirring at room temperature. After 30 min the mixture was centrifuged. The combined supernatant layers were vacuum-evaporated in a rotary distillation apparatus (Buchi) at 50°C to remove all solvents (including water) so that the phenol solidified at room temperature. LPS aggregates were precipitated from the phenol by warming and adding sufficient deionized water to bring the total suspension to 85% phenol (v/v). The aggregates were sedimented from the phenol solution by centrifugation (5000***g***, 1 h). The pelleted LPS was washed twice with 85% phenol and three times with absolute acetone. The final pellet was suspended in sufficient deionized water with sonication to form a particle-free, opalescent suspension. The pH of this suspension was adjusted to slightly more than pH 7 using a few μl of 0.0075 M triethanolamine and pH test strips. The suspension was dialyzed (same membrane as above) overnight at 4°C against several changes of deionized water. The resulting dialysate was finally lyophilized.

2.2.4. Ultracentrifugation

The dialyzed, lyophilized LPS was resuspended as above and ultracentrifuged at 77,000***g*** for 24 h at 4°C. The resulting pellet was resuspended in deionized water and lyophilized. The dried, highly purified LPS was stored at 4°C until used. Purity was confirmed by analyzing the LPS on polyacrylamide gel electrophoresis, according to the methods of Bell and Bell (1988).

The LPS was prepared for larval injections by adding aliquots of pyrogen-free water to dried LPS. LPS forms micelles of varying sizes in water; stable suspensions were formed by sonicating samples for approximately 10 min at 40 W with a probe sonicator (VibraCell, Danbury, CT) to produce micelles.

2.3. Injections and manipulations

Test larvae were injected with either the PLA_2 inhibitor dexamethasone [(11β,16α)-9-fluoro-11,17,21-trihydroxy-16-methylpregna-1,4-dione], the cyclooxygenase inhibitor ibuprofen [α-methyl-4-(2-methylpropyl)benzeneacetic acid], or the lipoxygenase inhibitor esculetin (6,7-dihydroxycoumarin), all purchased from BioMol (Plymouth Meeting, PA). In some experiments, larvae were injected with arachidonic acid (5,8,11,14-eicosatetraenoic acid). Control larvae were injected with the drug vehicle, 95% ethanol. All injections of pharmaceuticals, except esculetin, were in a standard volume of 2 μl. Esculetin was injected in a volume of 10 μl. Except in dose-response experiments, the pharmaceutical products were injected in dosages of 26 μg/larva and arachidonic acid (20:4n-6) was injected at 20 μg/larva.

2.4. General procedures

General procedures described in detail by Miller and Stanley (1998) are given here, and individual experiments are described in context in the Results section. Larvae were surface sterilized by swabbing with 95% ethanol. The insects were pretreated with a pharmaceutical product or control injection, then challenged with a standard dosage of 200 μg of purified LPS. The injections were done using a 26 gage, 0.5 in needle attached to a 50 μl syringe (Hamilton, Reno, NV). Microaggregation was assessed by applying 20 μl of hemolymph on to a Brightline hemacytometer (AO Instrument, Buffalo, NY). The number of cellular aggregates (operationally defined as a cluster of nine or more cells) in each sample was estimated by direct counting, using the grid system, under phase contrast optics.

2.5. Dose response for LPS

Individuals in five groups of larvae were separately injected with 0, 10, 50, 100 or 200 μg of LPS. After 1 h incubations, hemolymph was withdrawn and the number of cellular aggregates in each sample was estimated by direct counting as described above.

2.6. Time course of nodulation: influence of dexamethasone

Individuals in three groups of larvae were injected with 2 μl of ethanol or with 26 μg of dexamethasone in

2 μl of ethanol. A control group was injected with the drug vehicle, 2 μl ethanol, and then with 200 μg LPS. An experimental group was injected with dexamethasone, and then with 200 μg LPS. A third control group was included to assess the possible influence of the LPS vehicle, pyrogen-free water, on microaggreagation. This group was injected with 2 μl ethanol, and then with 20 μl water. The larvae were immediately injected with LPS as described. At 6, 12, 18, 30, 60, 90 and 120 min post-injection (PI), sub-groups of control and experimental insects were processed for assessing microaggregation as just described.

2.7. Dose-response curves for dexamethasone

Individuals in four groups of larvae were injected with 2 μl of ethanol, or 0.26, 2.6 or 26 μg of dexamethasone in 2 μl ethanol, and then challenged with a standard dosage of LPS. At 1 h PI, the larvae were processed for assessing microaggregation as just described.

2.8. Fatty acid rescue experiment

Individuals in two groups of larvae were injected with either 2 μl ethanol or 26 μg of dexamethasone in 2 μl of ethanol and challenged with LPS. Immediately after challenge, the dexamethasone-treated larvae were divided into two sub-groups. Individuals in one sub-group were treated with 20 μg arachidonic acid in 2 μl of ethanol. Another sub-group was treated with 2 μl of ethanol to control for the effects of the extra injection on nodulation. At 1 h PI, the larvae were processed for assessing microaggregation as just described.

2.9. Influence of other eicosanoid-biosynthesis inhibitors on nodulation

We divided larvae into groups and injected individuals in each group with either ibuprofen or the lipoxygenase inhibitor esculetin, all in standard dosages of 26 μg in 2 μl of ethanol. Control insects were injected with 10 μl of ethanol. Following injections, the larvae were challenged with LPS. At 1 h PI, the larvae were processed for assessing microaggregation.

2.10. Statistical analysis

Data on microaggregation were analyzed by analysis of variance (ANOVA) and significance was determined at $P<0.05$ using the Least Significant Difference (LSD) test.

3. Results

The beetle larvae formed hemocyte microaggregates in reaction to LPS injections by 1 h post-injection. The number of microaggregates increased in a linear way from about 0.4×10^5/ml in reaction to 5 μg of LPS/larva to about 0.8×10^5/ml in reaction to 100 μg/larva (Fig. 1). In this experiment, larger LPS dosages did not result in more microaggregates. With a view to maximizing microaggregation reactions in positive control experiments, we used 200 μg LPS treatments in subsequent experiments.

We investigated the influence of incubation time on the formation of hemocyte microaggregates in reaction to 200 μg LPS treatments (Fig. 2). The data represented by open circles show the normal time course of microaggregate formation in larvae challenged with LPS, then injected with ethanol to control for a second injection. We recorded negligible microaggregation at 6 min PI, which increased to about 0.3×10^5 at 12 min and then to nearly 1.5×10^5 microaggregates/ml hemolymph by 18 min PI. Longer incubations did not result in significantly greater numbers of microaggregates. The data represented by the solid circles show the influence of the eicosanoid-biosynthesis inhibitor, dexamethasone, on numbers of microaggregates in reaction to 200 mg LPS challenges. We recorded negligible numbers of microaggregates at 6 min PI, which increased to about 0.5×10^5 by 18 min PI; again, the numbers of microaggregates did not increase significantly with longer incubation periods. The third experiment (represented by solid triangles) was designed to control for the possibility that the handling and injecting procedures independently stimulate the microaggregate formation reaction. Fig. 2 shows that microaggregation reactions to injections of ethanol followed by pyrogen-free water yielded very low levels of microaggregate formation, which were not significantly

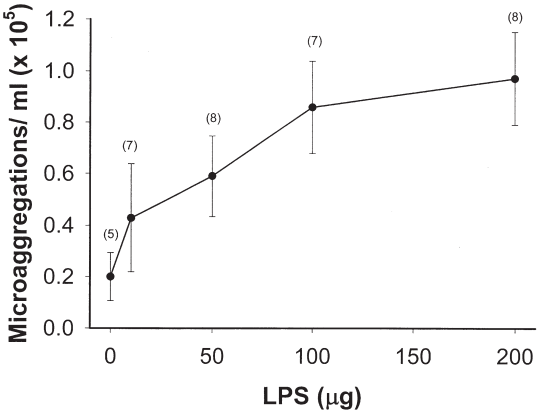

Fig. 1. The influence of LPS dosages, purified from the pathogenic bacterium *S. marcescens*, on numbers of microaggregates in the hemolymph from larvae of the beetle *Z. atratus*. The insects were injected with the amounts of LPS indicated, and after 1 h hemolymph samples were withdrawn and applied to a hemacytometer. Numbers of microaggregates were determined by direct counting. Data points represent mean numbers of microaggregates, and error bars indicate one standard error of the mean (SEM). Numbers in parenthesis indicate the number of individual insects in each experiment.

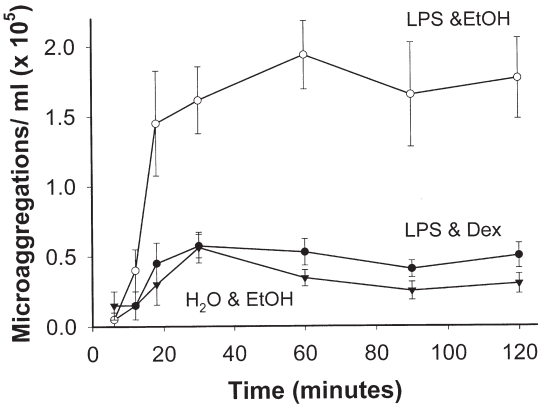

Fig. 2. Time course of microaggregation in larval beetles, *Z. atratus*, in response to injections of LPS (200 μg). Test insects were first injected with dexamethasone (Dex; solid circle), and control insects were first injected with ethanol (EtOH; open circles). Within 3–10 min, both groups of insects were then intrahemocoelically injected with 200 μg of LPS. An additional set of control insects (solid triangles) was treated with pyrogen-free water (H_2O) and with ethanol. At the indicated times PI, microaggregation was assessed. Each point indicates the mean number of microaggregates found in each insect, and the error bars represent one SEM. Each point represents the mean of five or more separate experiments.

different from the results with dexamethasone treatments.

Fig. 3 shows that the influence of dexamethasone on microaggregate formation was expressed in a dose-dependent way. Numbers of microaggregates declined from about 1.5×10^5 microaggregates/ml hemolymph in insects treated with the drug vehicle, ethanol, and then injected with LPS, to about 0.5×10^5 microaggregates/ml hemolymph in insects treated with the highest dosage.

The influence of dexamethasone on LPS-induced microaggregate formation was reversed by treating experimental insects with the eicosanoid precursor

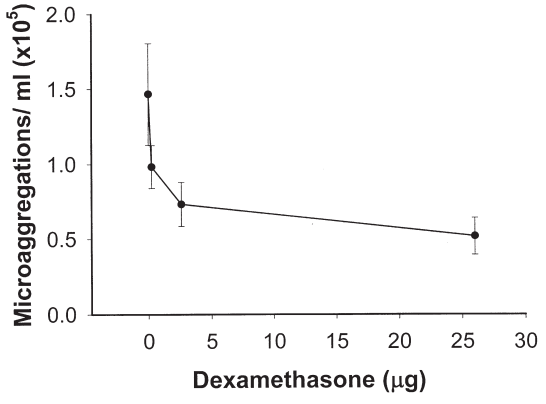

Fig. 3. Dose-response curve for the influence of dexamethasone on microaggregate formation. Larvae of *Z. atratus* were first injected with the doses of dexamethasone indicated and then intrahemocoelically injected with LPS. At 1 h PI, microaggregation was assessed. Each point (n=15) indicates the mean number of nodules found in each insect, and the error bars represent one SEM.

20:4n-6 (Fig. 4). In this experiment, ethanol-treated control insects yielded slightly over 1.5×10^5 microaggregates/ml hemolymph, while dexamethasone-treated insects yielded about 0.5×10^5 microaggregates/ml hemolymph. Another group of larvae were first treated with dexamethasone, then injected with LPS, and then given an injection of 20:4n-6. We recovered nearly 2.5×10^5 microaggregates/ml hemolymph from these insects. To control for the possibility that the third injection would somehow stimulate increased microaggregation, a fourth group of larvae was treated with dexamethasone, challenged with LPS, and then injected with the fatty acid vehicle, ethanol. At about 0.5×10^5 microaggregates/ml hemolymph, microaggregation in these insects was similar to that in the dexamethasone-treated counterparts.

We recorded the influence of ibuprofen, a specific cyclooxygenase inhibitor, and of esculetin, a 5- and 12-lipoxygenase inhibitor, on microaggregation reactions to LPS challenge (Fig. 5). Compared with controls, insects treated with either of these inhibitors yielded significantly reduced numbers of microaggregates, approximately 0.5×10^5 microaggregates/ml hemolymph, on par with the results of dexamethasone treatments.

4. Discussion

In this paper we report on two insights into cellular defense reactions in larvae of the tenebrionid beetle, *Z. atratus*. First, the larvae form hemocytic microaggre-

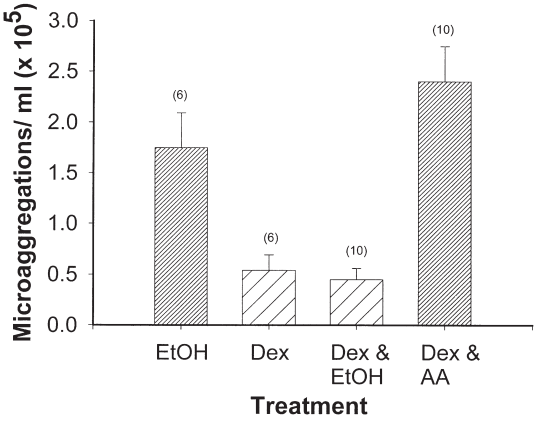

Fig. 4. Arachidonic acid reverses the effect of dexamethasone on microaggregate formation. Insects were treated with ethanol (EtOH) or dexamethasone (Dex) and then injected with LPS. Immediately after injection, test insects were treated with 20 μg of arachidonic acid (Dex+AA). Control insects were treated with dexamethasone and ethanol (Dex+EtOH). At 1 h PI, microaggregation was assessed. The height of histogram bars represents the mean number of microaggregates found in each insect, and the error bars represent one SEM. Histogram bars with the same fill pattern are not significantly different from each other (LSD, $P<0.01$). The numbers in parentheses above the error bars indicate the number of insects in each treatment.

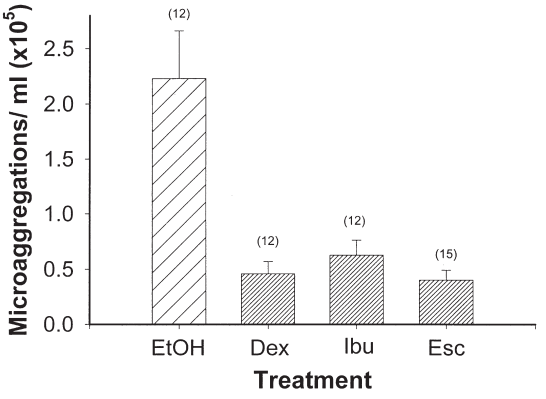

Fig. 5. Effects of treating *Z. atratus* larvae with individual eicosanoid biosynthesis inhibitors on microaggregate formation in response to intrahemocoelic injections of LPS. Test insects were first injected with 26 μg of either dexamethasone (Dex; PLA_2 inhibitor), esculetin (Esc; lipoxygenase inhibitor) or ibuprofen (Ibu). Control insects were first injected with ethanol (EtOH). Test and control insects were then intrahemocoelically injected with 200 μg of LPS. At 1 h PI, the insects were anesthetized on ice and nodulation was assessed. Each bar indicates the mean number of microaggregates found in each insect, and the error bars represent one SEM. Histogram bars with the same fill pattern are not significantly different from each other (LSD, $P<0.01$). The numbers in parentheses above the error bars indicate the number of insects in each treatment.

gates in reaction to LPS challenge and, second, the microaggregate reaction is mediated by eicosanoids. The idea that LPS can stimulate cellular defense reactions is well established in mammalian immunology (Weinstein et al., 1991), as well as in several invertebrate species, including mussels (Tunkijjanukij et al., 1997), annelid worms (Beschin et al., 1998), crustaceans (Lorenzon et al., 1997) and sea urchins (Smith et al., 1995). LPS-binding proteins have been detected in hemolymph from a variety of insect species, such as the silkworm, *B. mori* (Koizumi et al., 1997), the American cockroach, *Periplaneta americana* (Jomori et al., 1990), the blood-sucking bug, *Triatoma infestans* (Hypsa and Grubhoffer, 1995), and the fruitfly, *Ceratitis capitata* (Charalambidis et al., 1996). We infer that recognition of LPS as a component of certain bacterial cell surfaces is a general mechanism of cellular host defense systems. Less is known, however, about the biochemical events that mediate cellular reactions to LPS.

Several lines of evidence support our view that *Z. atratus* cellular reactions to purified LPS are mediated by eicosanoids. First, dexamethasone treatments severely curtailed LPS-induced microaggregate formation throughout the time course of the experiments. Second, the influence of dexamethasone on LPS-induced microaggregation was expressed in a dose-related manner. Third, the influence of dexamethasone on LPS-induced microaggregation was reversed by injecting arachidonic acid, an eicosanoid-precursor fatty acid, into dexamethasone-treated, LPS-challenged insects. Fourth, independent inhibition of the two major eicosanoid biosynthetic pathways, cyclooxygenase and lipoxygenase, substantially reduced microaggregation reactions to LPS challenge. These points have been discussed in detail elsewhere (Miller et al., 1996; Stanley, 2000), and this information is not repeated here. In the same vein, the pitfalls of relying on pharmaceutical inhibitors of eicosanoid biosynthesis as probes have been treated (Stanley-Samuelson, 1994). As posited for insect cellular reactions to bacterial infections (Stanley and Howard, 1998; Stanley, 2000), these findings strongly support the hypothesis that eicosanoids mediate cellular reactions to LPS challenge.

The significance of this idea lies in understanding the system of signal transduction pathways that operate between the recognition of a bacterial infection and the cellular defense reactions to the infection. As suggested in the Introduction, eicosanoids comprise one element of the system. The work of Downer and colleagues (Baines et al., 1992) shows that biogenic amines make up another element. Certainly there are others, and recent work with LPS is beginning to reveal some of them. Foukas et al. (1998) suggested that the Ras/mitogen-activated protein kinase pathway and integrin receptors are required for cellular internalization of LPS by hemocytes from *C. capitata*. So far, then, eicosanoids, biogenic amines and kinase pathways have been recognized as important elements in mediating cellular defense reactions to bacterial challenge. We anticipate that several other pathways will come to light in the near future. The emerging challenge, then, will be to develop a sophisticated understanding of multiple signal transduction systems in insect immunity.

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